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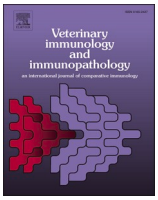

Design and development of multiepitope chimeric antigens by bioinformatic and bacterial based recombinant expression methods, with potential application for bovine tuberculosis serodiagnosis

Cassiano Martin Batista [a,d], Josir Laine Veschi [b], Vanessa Felipe de Souza [c], Leonardo Foti [a], Lea Chapaval Andri [d,*,1]

[a] Instituto Carlos Chagas/Fiocruz, Curitiba, Paraná, Brazil
[b] Empresa Brasileira de Pesquisa Agropecuária (Embrapa) Semiárido, Petrolina, Pernambuco, Brazil
[c] Embrapa Gado de Corte, Campo Grande, Mato Grosso do Sul, Brazil
[d] Embrapa Pecuária Sudeste, São Carlos, São Paulo, Brazil

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ABSTRACT

Bovine tuberculosis (bTB), which is caused by *Mycobacterium bovis,* is a single health concern, which causes economic losses, is a sanitary barrier and is a zoonotic concern. The golden-pattern intradermic tests have low sensitivity of about 50%. To fix this sensitivity problem, immunoassays could be a powerful tool. However, few studies produced antigens for bTB immunoassays, which needs improvements. Aim of this study was to produce multiepitope chimeric antigens (MCA) to use for bTB diagnosis. To achieve MCA design and development, extensive bibliographic search, antigenic epitope prediction, specificity, hydrophobicity, and 3D structure modeling analyses were performed, as well as cloning, expression and purification. Seven epitopes from four different target proteins (MPB-70, MPB-83, ESAT-6 and GroEL) were combined in five chimeras containing five repetitions of each epitope to enhance antibodies affinity. 3D predicted models revealed that all chimeras have a high percentage of disorder, which could enhance antibody recognition, although taking to protein instability. Each chimera was cloned into pET28a (+) expression plasmids and expressed in six *Escherichia coli* expression strains. Chimeras 3, 4 and 5 could be solubilized in 8 M urea and purified by ion exchange affinity chromatography. Against bTB positive and negative sera, purified chimera 5 had the best results in indirect dot blot and ELISA, as well as in lateral flow dot blot immunoassay. In conclusion, chimera 5, an MPB-83 containing MCA, could be used for further studies, aimed to develop a serologic or rapid test for bTB diagnosis.

1. Introduction

Mycobacterium bovis (*M. bovis*), is a Mycobacterium that can cause TB disease in animals and in humans, being a major zoonotic disease, and cattle are the main source of infection for humans (McDaniel et al., 2014; Borham et al., 2022). *M. bovis* is most commonly found in cattle and other animals such as bison, elk, and deer. In humans, *M. bovis* causes TB disease that can affect the lungs, lymph nodes, and other parts of the body. If the patient is immunocompromised, the disease could be moderate to severe (LoBue et al., 2010). Because of those facts it is mandatory to the farmers/veterinarians to control this infection and do contention (e.g., investing in diagnosis).

Intradermal tests are yet considered as the gold-standard for bTB diagnosis (Lahuerta-Marin et al., 2016). It consists of injection of Purified Protein Derivative (PPD), which is a *M. tuberculosis* protein extract, into the skin of the animals suspected of being infected. The reading is done in three days, the reaction should be measured in millimeters of the induration (firm swelling) and have a sensitivity of about 50% and the interpretation can be altered depending on the level of TB in the area or other disease factors. In addition, the result could be masked by 3 months passed vaccinated animals (Palmer et al., 2010), and the herd owner must wait 60 days after the first intradermal test before a follow-up, or retest, can be carried out, as mentioned in Bovine Tuberculosis Eradication USDA Reference Guide (2005). Based on this,

* Correspondence to: Embrapa Pecuária Sudeste, Microbiologia Molecular/Diagnósticos Rápidos, PAB, Brazil.
E-mail address: lea.chapaval@embrapa.br (L.C. Andri).
[1] Rod. Washington Luiz, km 234 - Caixa Postal 33913560-970 - São Carlos, São Paulo, Brasil

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serological or molecular tests could be developed to turn bTB diagnosis faster, sensible, precise and with better coverage.

Souza and colleagues (2012) selected a chimera of hydrophilic domains of ESAT-6/ MPB-70/ MPB-83 as a potential antigen for serological bTB diagnosis. The same group, in a more recent study (2019), showed that this chimera complements the diagnostic coverage by intradermal tests. At the same time, Alonso and colleagues (2021) compared three bTB antigens (MPB-83, ESAT-6 and CFP-10) in a lateral flow rapid test platform and concluded that it is possible to develop a lateral test for bTB diagnosis. Fresco-Taboada and colleagues (2019) also developed a lateral flow test, based only in MPB-83, with 83% sensitivity and 97% specificity, but for identification in wild boar samples, a *M. bovis* wild reservoir (Fresco-Taboada et al., 2019). Hekal and colleagues (2022) performed an extensive comparative study of a great number of different kinds of diagnostic tests, such as intradermal test, conventional tests (*M. bovis* isolation and PCR confirmation), ELISA and the most recent developed, the IFN-gamma test, which was the most sensitive. Interestingly, an immunoassay using serum samples based on polypeptide antigens (89.81% of positivity) was better than PPD (87.03% of positivity) (Hekal et al., 2022). Based on this background, immunogenic antigens development, such as multiepitope chimeric antigens (MCA), with epitope repeats to improve diagnostic potential of these engineering target proteins, are an interesting approach for development of diagnostic immunoassays.

Aim of this study was to develop MCA to use them for bTB diagnosis. To do that, we did extensive bibliographic search, sequence analysis, 3D structure prediction, cloning, bacterial expression, protein purification and different immunoassays against bTB positive and negative sera. As a major result, we found a chimera, named Chimera 5 (C5), with potential for further application studies for the development of serological tests, such as ELISA and/or lateral flow platforms for bovine tuberculosis serodiagnosis.

2. Materials and methods

2.1. Reagents

Kanamycin, Isopropyl β-d-1-tiogalactopiranosídeo (IPTG), Phosphate Buffer Saline pH 7.4 (PBS), Urea, Monosodium phosphate, Imidazole, Phosphate Buffer Saline with Bovine Serum Albumin 10% (PBS/BSA 10%), 3,3′,5,5′-tetrametilbenzidine (TMB), Sodium carbonate, Sodium bicarbonate, Tris-hydrochloride (Tris-HCl) and Tween 20 were purchased from Merck, USA. Luria-Bertani broth media (LB), microBCA kit, Page Ruler protein prestained ladder 10–250 kDa, goat IgG anti-bovine antibody conjugated to Horseradish peroxidase (HRP) and goat IgG anti-bovine unconjugated antibody were purchased from Thermo Fisher, USA. NiNTA-agarose resin and miniprep kit were purchased from Qiagen, Japan. Secondary antibody goat anti-mouse IgG 680 was purchased from Lycor, USA. Coomassie G-250 was purchased from BIORAD, USA. Mouse anti-6xhis tag antibody was purchased from Genscript, USA, and tablet of protease inhibitor EDTA-Free, EasyPack, was purchased from Roche, Swiss.

2.2. Bibliographic search, targets choice and sequence analysis

To design potential antigens to develop MCA, a bibliographic search was done in PUBMED (NCBI, USA), with the key words "*M. bovis* proteomic, immunodominant antigens and antigen prediction". Targets choice (Five proteins) was done by antigen frequency and sequence analysis, which were epitope prediction using the Antigenic tool of Immunomedicine Group, Universidad Complutense Madrid (http://imed.med.ucm.es/Tools/antigenic.pl, Spain), homology by BLASTp (NCBI, USA) and hydrophobic prediction using Peptide 2.0 software (https://www.peptide2.com/ N_peptide_hydrophobicity_hydrophilicity.php, USA). Only epitopes with Average Antigenic Propensity above 1.0, with homology and hydrophobicity below 50% were considered targets in the analysis. 3D protein structures were predicted in Phyre 2.0 software (Kelley et al., 2015), with normal and/or intensive modeling types.

2.3. Multiepitope chimeric antigens design and cloning

After sequence analysis, MCA construction and production were performed. Briefly, five epitope repeats in a 3–3 protein combination scheme, to exclude one of the four proteins, were designed using SnapGene software (Dotmatics, USA). After reverse translation in "The Manipulation Suite" software (https://www.bioinformatics.org/sms2/rev_trans.html, USA) and codon optimization on GenSmart software (Genscript, USA), five chimeric constructions were performed.

Synthetic chimeric DNA sequences were cloned into pET28a (+) (Twist Bioscience, USA), with *Nco*I and *Xho*I restriction sites, resistance to kanamycin, lactose operon for recombinant protein expression with IPTG induction and 6xhis tagged at the C terminus of the produced protein. After receiving the sequenced clones (25 ng/μL), 25 ng of each chimeric construction were used to expand in *Escherichia coli* TOP10 strain and plasmid purification was did by Miraprep (Pronobis et al. 2016), with miniprep kit (Qiagen, Japan) and by adding ethanol 100% (v:v) in the supernatant of neutralization centrifugation step of the kit.

2.4. Expression and purification of multiepitope chimeric antigens

For expression of chimeric constructs, 200 ng of each clone were heat-shock transformed into six *E. coli* expression strains: BL21 (DE3) pLysS, BL21 (DE3) Star, BL21 (DE3) ΔSlyD pRARE, Rosetta Gami 2 (DE3), BL21 C41+ (DE3) and BL21 C43+ (DE3).

A first expression test was done to find chimeric solubility in *E. coli*. Each clone was inoculated with 10 mL of LB broth medium with 50 μg/mL of kanamycin, and protein induction was performed in optimal optical density (OD) of about 0.6, for four hours at 37° C and 200 rpm in the presence of 1 mM of IPTG. Not Induced (NI) and Whole Fraction (WF) were collected. After that period, the bacterial pellets were washed twice in PBS and resuspended in PBS to lysis by sonication for 5 times, 10 seconds, and potency of 3% in a ultrasonicator equipment Q700 (QSonica, USA) with 3 mm tip. The samples were then centrifuged for 5 minutes at 20000 x g. After centrifugation, supernatants were the Soluble Fraction (SF), and pellets were the Insoluble Fraction (IF). Aliquots of all fractions were done and used for quality control by denaturing SDS-PAGE 12% according to Laemmli (1970), incubated with Coomassie G-250 and documented in a photodocumenter L-pix X (Loccus, Brazil) and anti-6xhis tag western blot according to Towbin and colleagues (1979) (Towbin et al., 1979), which was diluted in PBS-Tween 0.05% and 5% milk according to the fabricant (1:5000), as well as secondary antibody anti-mouse IgG 680, 1:10000, and revelation of fluorescence was done in the Odyssey 9120 reader (Lycor, USA).

To solubilize IF and facilitate chimeric antigens purification by ion exchange affinity chromatography (IMAC), each clone of BL21 (DE3) Star with inserts was inoculated with 100 mL of LB in the same conditions as described below. After SF and IF separation by sonication, IF was resuspended in 2 mL of Buffer 1 (500 mM NaCl, 20 mM Tris-HCl, 8 M Urea, 10% Glycerol, and a tablet of protease inhibitor, pH 8.0), sonicated for 5 times, 15 seconds, potency of 5%, and incubated for 2 h at 4° C and 50 rpm (Santos and Souza, 2018). The solution was centrifuged for 10 min, 20000 x g and 4° C and the collected supernatant was being the Insoluble Fraction Solubilized (IF-S) and the pellet was being the Insoluble Fraction Not Solubilized (IF-NS). Aliquots of all process steps were done for quality control by SDS-PAGE 12% (Laemmli, 1970).

Purification was done by IMAC purification using NiNTA-agarose resin (Skerra et al., 1991), according to fabricant instructions. In a 1.5 mL microtube, IF-S of each chimeric antigen was incubated with NiNTA resin for 1 hour at 4° C and 50 rpm, centrifuged at 1500 rpm for 30 seconds (supernatant: Flow Through), and then incubated twice with Buffer 2 (50 mM Monosodium phosphate (NaH2PO4); 100 mM NaCl,

20 mM Imidazole, pH 8.0), centrifuged 1500 rpm for 30 seconds (Supernatants: Washed fraction), and then incubated with Buffer 3 (Buffer 2 with 100 mM Imidazole), centrifuged 1500 rpm for 30 seconds (Supernatants: Elution 1) and, finally, incubated with Buffer 4 (Buffer 2 with 250 mM Imidazole), centrifuged 1500 rpm for 30 seconds (Supernatants: Elution 2). In addition, eluted fractions from different purification tests were incubated together in a NiNTA Spin column and centrifuged 1500 rpm for 1 minute to enhance purified protein yielding. Protein concentration of purified chimeric antigens were performed by microBCA kit (Walker, 1994), according to the fabricant. Aliquots of all steps were done to the quality control by SDS-PAGE 12% (Laemmli, 1970) and anti-6xhis tag western blot (Towbin et al., 1979) and both were revealed in the same conditions as described below. In detail, 1 μg of purified with 250 mM Imidazole proteins in spin columns were added to the acrylamide gels.

2.5. Bovine tuberculosis serum samples

In this study, we used thirteen for both negative sera from group A (bTB-free herd) and positive sera from group B (bTB-positive herd) from the bTB sera-panel described elsewhere (Souza et al., 2019), all previously tested by intradermal test, ELISA, confirmatory bacterial culture and PCR.

2.6. Indirect dot blot of purified multiepitope chimeric antigens against bovine tuberculosis serum pool samples

To test if the purified MCA could recognize negative or positive bovine sera samples for bTB, a dot blot was performed according to Newman and collaborators (1981) (Newman et al., 1981). First, 1 μg of each purified chimera was incubated with a nitrocellulose membrane (Amershan, USA) and let dry for about 16 hours at 25° C. As a negative control, the same volume of PBS was incubated in the same conditions. After that, the membranes were blocked with PBS-Tween 0.05% with 5% of dry milk (Blocking solution) for 1 h at 25° C and then incubated, separately, with bovine sera of negative or positive pools of bTB, diluted at 1: 1000 in blocking solution, for 1 h hour at 25° C. After three washes with PBS-Tween 0.05%, membranes were incubated with an IgG anti-bovine antibody conjugated to HRP, diluted at 1:5000 in blocking solution, for 1 h hour at 25° C. Membranes with the MCA were incubated only with this conjugated antibody as a control. After three washes with PBS Tween 0.05%, the membranes were incubated with luminol/peroxide (v/v) solution and revealed in the iBright 1500 equipment (Thermo Fisher, USA), using the Smart Exposure tool.

2.7. Indirect ELISA of purified multiepitope chimeric antigens against bovine tuberculosis serum pool samples

To standardize the indirect ELISA immunoassay (Engvall and Perlmann, 1971), different C3–5 antigens concentrations (from 62,5 ng to 8 μg/ well) were adsorbed with carbonate/bicarbonate 0.05 M buffer (Coating Buffer) for 16 hours at 4° C in a 96 wells plate 65511 T (Cralplast, Brazil). After blocking with PBS/BSA 1% (Diluted from PBS/BSA 10% with PBS) for 1 hour at 37°C and four washes with PBS/Tween 0.05%, wells were incubated with different negative/-positive bTB bovine pool sera, diluted in with PBS/BSA 1% (1:100, 1:500 and 1:1000). After four washes with PBS/Tween 0.05%, wells were incubated with an IgG anti-bovine antibody conjugated to HRP, diluted 1:5000 in PBS/BSA 1%, for 1 h hour at 37° C. After four washes with PBS/Tween 0.05%, wells were incubated with TMB prepared solution for 10 minutes at 25° C, neutralized with 5 N hydrochloric acid, read at 450 nm absorbance in a H1M2F Synergy spectrophotometer (Biotek, USA), and analyzed in an Excel 2301 version (Microsoft, USA).

To test diagnostic potential of purified chimera 5, wells were incubated with 62.5 ng of this antigen diluted in the Coating Buffer for 16 hours at 4° C in 96 wells plate. After blocking with PBS/BSA 1% for 1 hour at 37°C and four washes with PBS/Tween 0.05%, wells were incubated separately with negative and positive bTB bovine pool sera as controls and with thirteen positive and negative bTB bovine sera diluted with PBS/BSA 1% at 1:500 for 1 hour at 37° C. After four washes with PBS/Tween 0.05%, wells were incubated with an IgG anti-bovine antibody conjugated to HRP, diluted 1:5000 in PBS/BSA 1%, for 1 h hour at 37° C. A control incubating only secondary antibody was performed. After four washes with PBS/Tween 0.05%, wells were incubated with TMB prepared solution for 10 minutes at 25° C, neutralized with 5 N hydrochloric acid; read at 450 nm absorbance in a H1M2F Synergy spectrophotometer and analyzed in an Excel 2301 version. The cut-off was calculated according to anti-hepatitis B ELISA (Labpedia, Brazil) as "$Co = (xNC + xPC)X0.4$". All absorbances with ratio of normalized absorbance with cut off up to 1.0 were considered negative, and above 1.0 positive.

2.8. Lateral flow dot blot of purified C5 multiepitope chimeric antigen against bovine tuberculosis serum pool samples

To test purified C5 diagnostic potential for lateral flow rapid tests (Yalow and Berson, 1960), 345 ng of this antigen was incubated at the "Test line" and 1 μg of anti-IgG bovine unconjugated antibody was incubated in the "Control line" of a lateral flow nitrocellulose membrane (Sartorius 180, USA) and let dry for 15 min at 37° C. After that, 5 μL of negative or positive pooled sera and 100 μL Running Buffer (0.2 M Tris HCl/ 0.01% Tween 20, pH 8.0) were added in the Sample Pad (Sartorius, USA). The samples passed through the glass fiber Conjugate Pad with 0.80 μL/mm of Protein A - 40 nm Colloidal Gold impregnated, and the complex passes into nitrocellulose membrane containing sample and control lines. The runs were of about 20 min and the reading was visual and with photos. The cassettes with Protein A - 40 nm Colloidal Gold impregnated to the conjugate pad, sample pad, nitrocellulose membrane and waste pad were gently ceded by Dr. Edmilson Domingues da Silva, director of LATED Laboratory (Biomanguinhos, Fiocruz, Rio de Janeiro, Brazil).

3. Results

3.1. Bibliographic search, target definition and analysis for chimeric constructions

As a result of the bibliographic search, we based our work in two references (Beltrán et al., 2011; Infantes-Lorenzo et al., 2017). In the former study, Beltrán and colleagues (2011) identified four proteins by expression library immunization as immunodominant antigens. In the second study, Infantes-Lorenzo and colleagues (2017) identified, by proteomic characterization of bovine (bPPD), avian (aPPD) and immunopurified bovine fraction (P22) protein extracts, the ten most abundant proteins in the P22 as the most specific to bTB serodiagnosis. We, therefore, choose five potential proteins for further sequence analysis (Table 1): MPB-70, MPB-83, GroEL, ESAT-6 and Elongation Factor Tu (EfTU). After epitope prediction, specificity, and hydrophobicity analysis, only epitopes 3 and 4 for MPB-70, epitope 10 for MPB-83, epitope 6 for GroEL and epitopes 1, 3 and 4 for ESAT-6, were selected (Table 1). Unfortunately, EfTU had high conservation of 73% with its mitochondrial homologue in *Bos taurus*, as well as high hydrophobicity, and no epitopes were selected from this protein (Table 1).

For MCA constructions, reverse translation of selected epitopes were done and nucleotide DNA sequences were combined in the following synthetic sequences produced and cloned into pET28a (+), according to methods section: 1) All epitopes (Chimera 1, named as C1); 2) Epitopes from MPB-70, MPB-83 and GroEL (Chimera 2, named as C2); 3) Epitopes from MPB-70, MPB-83 and ESAT-6 (Chimera 3, named as C3); 4) Epitopes from MPB-70, ESAT-6 and GroEL (Chimera 4, named as C4) and; 5) Epitopes from MPB-83, ESAT-6 and GroEL (Chimera 5, named as C5).

MCA 3D structures were predicted by Phyre 2 software (Kelley et al.,

Table 1
Target proteins choice and sequence analysis for construction of MCA.

Protein/ Reference	Number of predicted epitopes	Specificity (excluded epitopes)	Hydrophobicity (excluded epitopes)	Selected Epitope (s)
MPB-70/ (Infantes-Lorenzo et al. 2017)	10	4	1, 2, 5, 7, 8, 9, 10	3, 6
MPB-83 / (Infantes-Lorenzo et al. 2017)	13	8	1, 2, 3, 4, 5, 6, 7, 9, 11, 12, 13	10
ESAT-6/ (Infantes-Lorenzo et al. 2017)	4	2	-	1, 3, 4
GroEL/ (Beltrán et al. 2011; Infantes-Lorenzo et al. 2017)	21	1, 2, 3, 9, 11, 12, 13, 16, 17	5, 7, 8, 10, 14, 15, 18, 19, 20, 21	6
EfTU/ (Beltrán et al. 2011; Infantes-Lorenzo et al. 2017)	20	All	-	-

2015) (Fig. S1) and showed that they had the respectively coverage and confidence percentages: C1 (89 and 98%, final model), C3 (99 and 94%, final model), C4 (97% for both, final model) and C5 (91 and 90.6%, top model green fluorescent protein/cytoplasmic dynein 2 heavy chain protein). Unfortunately, C2 had no hits with >5% confidence and any modeling were predicted. Furthermore, all chimeras had high percentages for predicted protein disorder: C1 (58%), C3 (81%), C4 (67%) and C5 (64%).

3.2. Expression and purification of multiepitope chimeric antigens

To standardize MCA expression and solubility, the pET28a (+) clones were transformed into *E. coli* expression strains panel, as mentioned in the "Methods section". After IPTG induction in a 10 mL bacterial inoculum and bacterial lysis, an SDS-PAGE and anti-6xhis western blot were performed. As a result, BL21 (DE3) Star and BL21 (DE3) ΔSlyD pRARE expressed all chimeras (Table 2) in the expected predict molecular mass (C1: 53 kDa, C2: 21 kDa, C3: 30 kDa, C4: 44 kDa and C5: 25 kDa), with C2 only detected by anti-6xhis western blot and C3 with two polypeptide recognitions: one of about 15 kDa and other of about 35 kDa, with the last one detected only by anti-6xhis western blot. BL21 (DE3) C41+ expressed all chimeras, with exception of C2. BL21 (DE3) pLysS only expressed C1, C3 and C4. BL21 (DE3) C43+ only expressed C3, C4 and C5. Rosetta Gami 2 only expressed C2 and C3, with C2 only detected by anti-6xhis western blot (Table 2). As a general protein solubility result, all expressed MCA were in insoluble fraction (IF) of the bacterial extract. Based on these results, we decided to not work with C2 in the next experiments.

Before purification, IF of C1, C3, C4 and C5 from a 100 mL *E. coli* BL21(DE3) Star cultures were solubilized with 8 M urea. C3–5 could be solubilized (IF-S, data not shown). Of note, C1 presented protein instability in SDS-PAGE (Data not shown), with a degradation profile. Due to this instability, we followed up with C3–5 purification by IMAC with NiNTA-agarose resin. After two pilot purification steps in 1.5 mL microtubes and a third step in a spin column with eluted fractions and resins from the pilots, we could observe by SDS-PAGE that the best elution of C3–5 was with 250 mM imidazole, with anti-6xhis specific recognition (Fig. 1).

Table 2
Standardization of MCA expression in *Escherichia coli* expression strains, analyzed by SDS-PAGE and anti-6xhis western blot. ND: Not detected; Only WB: Detected only by anti-6xhis WB; +, ++, +++ and ++++: Ascending order of protein expression level.

E. coli strain	C1	C2	C3	C4	C5
BL21 (DE3) Star	++++	Only WB	++++	++++	++++
BL21 (DE3) ΔSlyD pRARE	+++	Only WB	+++	+++	+++
BL21 (DE3) C41+	+	ND	++	++	+
BL21 (DE3) pLysS	+	ND	++	++	ND
BL21 (DE3) C43+	ND	ND	++	++	+
Rosetta Gami 2	ND	Only WB	+++	ND	ND

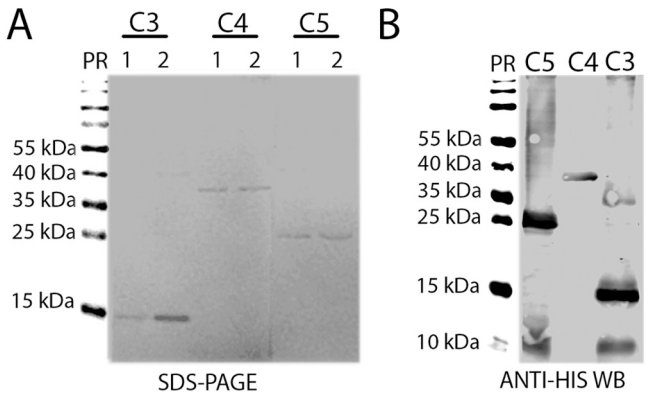

Fig. 1. Purification of multiepitope chimeric antigens C3-5 analyzed by SDS-PAGE 12% and anti-6xhis western blot. A) SDS-PAGE 12%; B) Anti-6xhis western blot 250 mM imidazole elution. PR: Page Ruler protein prestained ladder; 1: elution with 250 mM imidazole; 2) NiNTA resin.

3.3. Indirect dot blot of purified multiepitope chimeric antigens against positive and negative sera from bovine tuberculosis

To test if positive or negative bTB bovine serum samples could recognize or not with a specific response to the purified MCA, an indirect dot blot was performed. The result showed that only purified C5 had expected results, with a positive reaction only for the positive bTB pooled serum (Fig. 2), despite purified C3 and C4 nonspecific results as negative bTB serum having signal (Data not shown).

3.4. Indirect ELISA of multiepitope chimeric antigens against positive and negative sera from bovine tuberculosis

First, standardization of an indirect ELISA was performed. Purified C3 and, mostly, C5, showed ratios about 2 between positive and negative pooled bTB bovine sera absorbances (Data not shown), mainly for 250, 125.5 and 62.25 ng of purified proteins and 1:500 and 1:1000 for sera dilutions. Purified C4 did not have that profile: there was no difference between positive and negative pooled bTB bovine sera absorbances (Data not shown).

Due to present the best ratio between positive and negative pooled

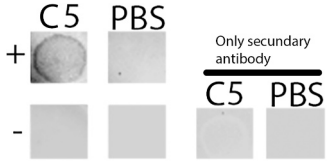

Fig. 2. Indirect dot blot of purified Chimera 5 multiepitope chimeric antigen against positive and negative sera from bovine tuberculosis. C5: purified C5 incubated with positive and negative bTB sera; PBS: purified C5 incubated with positive and negative bTB sera; Only secondary antibody: anti-bovine IgG HRP incubated with purified C5 or PBS without bTB sera incubation.

bTB bovine sera absorbances, purified C5 was challenged in a bovine bTB serum test, using 62.25 ng of the antigen and serum dilution at 1:500. As a result, after normalization by decreasing the signal of the only secondary antibody absorbance mean from the obtained absorbance and dividing normalized absorbance by cut-off, which was calculated in 0.292, 10/13 (73.92%) were positive and 12/13 (92.31%) were negative (Table 3).

3.5. Lateral flow dot blot of purified chimera 5 multiepitope chimeric antigen against positive and negative sera from bovine tuberculosis

To achieve if purified C5 could be used in rapid test immunoassays, a dot blot in the lateral flow platform was performed. After the running step, a positive reaction could be observed in the "Test line" against positive pool of bTB sera (Fig. 3), as well in the "Control line" (Anti-bovine IgG antibody) from both positive and negative sera, as expected. On the other hand, no or weak reaction was observed for the negative pool of bTB sera (Fig. 3).

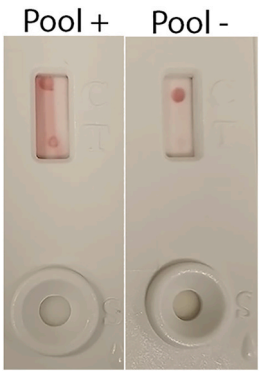

Fig. 3. Lateral flow dot blot of purified multiepitope chimeric antigen chimera 5 against positive and negative sera from bovine tuberculosis. Up signal: "Control line (C)" with anti-bovine IgG unconjugated antibody, down signal: "Test line (T)" with purified chimera 5. S: sample pad, where the sample and running buffer were deposited.

4. Discussion

Bovine tuberculosis (bTB) is a unique health concern, representing economic losses, sanitary barriers, and zoonotic concerns (McDaniel et al., 2014; Borham et al., 2022) and complete elimination of the disease is complicated by persistent infection of wild animals. Because of this fact, it is mandatory to the farmers/veterinarians to control this infection and do contention. Increase of diagnostic coverage is a crucial step. Intradermal tests are yet considered as the gold-standard in bTB diagnosis (Lahuerta-Marin et al., 2016). However, this test has a sensitivity of about 50% (Lahuerta-Marin et al., 2016). On the other hand, serological or molecular tests could be developed to turn bTB diagnosis faster, sensible, precise and with better coverage.

There are some antigens with potential diagnosis for bTB usage in the literature (Souza et al., 2012; Souza et al., 2019; Fresco Taboada et al., 2019; Garbaccio et al., 2019; Alonso et al., 2021; Zhu et al., 2022; Sridhara et al., 2022; Griffa et al., 2020). MCA are used for diagnosis of various infectious diseases (AnandaRao et al., 2005; Lin et al., 2008; Ribeiro et al., 2019; Gaikwad et al., 2019; Yin et al., 2020; Jameie et al., 2020), including bTB (Lyashchenko et al., 2021). As an increment, the epitope could be designed in various repeats, increasing the possibility of antibody ligation (Gaikwad et al., 2019). In this line of thought, in this study we developed MCA with five repeats of each predicted epitope to enhance antibody ligation.

To design and develop that, we designed a pipeline based in three pillars: 1) Bibliographic search in PUBMED; 2) Analysis of the targets in terms of its predicted immunogenicity, conservation, hydrophobicity and 3D structure predictions and, 3) Production of MCA (reverse translation, cloning, expression, purification) and downstream applications such as immunoassays against positive and negative bovine sera of TB samples.

After bibliographic search, we selected five potential proteins for further *in silico* sequence analysis: MPB-70, MPB-83, GroEL, ESAT-6 and Elongation Factor Tu (EfTU). After epitope prediction, specificity, and hydrophobicity analysis, only epitopes from MPB-70, MPB-83, GroEL and ESAT-6 were selected. Unfortunately, EfTU had high conservation as well as high hydrophobicity, and no epitopes were selected from this protein.

MCA 3D structure models were predicted and it was possible to observe that all chimeras had high percentages of disorder and are linear, when proteins have no secondary or tertiary structures, varying from 58% to 81% (Fig. S1). Interestingly, although being unstable for degradation and proteolysis (Suskiewicz et al., 2011), intrinsically disordered proteins are largely present in some pathogenic organisms and are *bona fide* targets of antibody recognition (MacRaild et al., 2016). So, this characteristic could be used to optimize serodiagnosis platform tests, such as ELISA and lateral flow. Unfortunately, no 3D model could be predicted for C2. However, there is a great chance of this chimera also having a high disorder percentage.

To standardize MCA expression and solubility, the pET28a (+) inserts were transformed into an *E. coli* strains expression panel, induced by IPTG, and lysed by sonication. BL21 (DE3) Star and BL21 (DE3) ΔSlyD pRARE expressed all chimeras with C2 only detected by anti-6xhis western blot, which could be protein instability, due to its possible disordered structure (Suskiewicz et al., 2011) (Table 2) and C3 presented two oligopeptides: 15 and 35 kDa, which could be due to proteolytic activity in consequence to its disordered structure (Suskiewicz et al., 2011). All expressed MCA were in insoluble fraction (IF) of the bacterial extract. Based on these results, we decided to not work with C2 due to low yielding and work only with BL21 (DE3) Star strain in the

Table 3
Indirect ELISA of multiepitope chimeric antigens against positive and negative sera from bovine tuberculosis. PC: Positive control; NC: Negative control. Normalization: sample absorbance means minus only secondary absorbance mean. Ratio: normalized absorbance/ cut-off. All ratios up to 1.0 were considered as negative and above 1.0 as positive.

Sample	Absorbance Average	Normalization	Ratio	Expected Result	Obtained Result
PC	1.1280	0.6035	2.0668	+	+
NC	0.6510	0.1265	0.4332	-	-
1	1.2140	0.6895	2.3613	+	+
2	1.3200	0.7955	2.7243	+	+
3	1.7180	1.1935	4.0873	+	+
4	0.7800	0.2555	0.8750	+	-
5	1.2580	0.7405	2.5360	+	+
6	1.2650	0.7405	2.5360	+	+
7	1.1960	0.6715	2.2997	+	+
8	0.8170	0.2925	1.0017	+	+
9	0.7360	0.2115	0.7243	+	-
10	0.7520	0.2275	0.7791	+	-
11	1.0970	0.5725	1.9606	+	+
12	1.1430	0.6185	2.1182	+	+
13	1.0040	0.4795	1.6421	+	+
14	0.7880	0.2635	0.9024	-	-
15	0.6870	0.1625	0.5565	-	-
16	0.6420	0.1175	0.4024	-	-
17	0.7910	0.2665	0.9127	-	-
18	0.7510	0.2265	0.7757	-	-
19	0.6630	0.1385	0.4743	-	-
20	0.6850	0.1605	0.5497	-	-
21	0.8700	0.3455	1.1832	-	+
22	0.7250	0.2005	0.6866	-	-
23	0.7040	0.1795	0.6147	-	-
24	0.6020	0.0775	0.2654	-	-
25	0.6770	0.1525	0.5223	-	-
26	0.7950	0.2705	0.9264	-	-

next experiments, because this strain had the best results.

Before purification of MCA, IF of C1, C3–5 were incubated with 8 M urea and C3–5 could be solubilized (IF-S). As mentioned above, C1 presented protein instability in SDS-PAGE, with a degradation profile, which might be caused by its disordered structure (Suskiewicz et al. 2011). Due to this instability, we followed up with C3–5 purification by affinity chromatography with NiNTA-agarose resin. As a result, we could observe by SDS-PAGE that the three antigens eluted with 250 mM imidazole, with anti-6xhis specific recognition (Fig. 1), with C3 presenting the same proteolysis profile described above.

To test if positive or negative bTB bovine serum samples could recognize or not with specific response to the purified MCA, an anti-bTB pooled sera western blot was first performed, and no positive results were observed in the tested conditions (pooled sera diluted at 1:100 or 1:500) (data not shown). Heating these chimeras could affect antibody recognition by changes in its structure and instability due to protein disorder (MacRaild et al., 2016). However, an indirect dot blot was performed, due to not affecting protein structure and the simplicity of this assay. Only purified chimera 5 had the expected results, with a positive reaction only for the positive bTB pooled serum (Fig. 2). This assay suggested that purified C5 is the most specific MCA.

To achieve purified MCA could be used in ELISA serological platform tests, first an indirect ELISA standardization was performed and purified C5 had the best results due to showing the best ratio between positive and negative pooled bTB pooled bovine sera absorbances. In addition, purified C5 was selected for a bovine serum challenge indirect ELISA test and 10/13 (73.92%) were true positive and 12/13 (92.31%) were true negative (Table 3). Despite needing improvements, such as secondary antibody dilution and to test more serum samples, this result suggests that purified C5 could be used for further studies to the development of ELISA serological tests for bTB.

To achieve if purified C5 could be used in rapid test immunoassays, a dot in the lateral flow immunochromatographic platform was performed. After the running step, a positive reaction could be observed in the “Test line” against a positive pool of bTB sera (Fig. 3). On the other hand, no or low reaction was observed for the negative pool of bTB sera (Fig. 3), suggesting that purified C5 could be used for rapid test development.

It is important to mention some limitations of this study: 1) while the multiepitope chimeric antigens were characterized biochemically, there was no functional performance of each protein component included in the fusion attempted to analytically demonstrate antibody detection by the respective epitopes within the chimera; 2) Some experiments using pooled sera instead of individual samples could not provide important data on the antibody detection rates; 3) Staphylococcal protein A used in the lateral-flow assay is not the most sensitive detector of bovine IgG, as it binds only to one of the two IgG subclasses in cattle. In contrast, streptococcal protein G shows a significantly stronger binding affinity for bovine IgG (Akerström et al., 1985). Future studies focusing on resolving these limitations could be crucial to better understand the results obtained in this work, and could provide the basis for the development of a new rapid test for serodiagnosis of bTB.

When comparing the constitution of C3–5 multiepitope chimeras, the only difference is the absence of MPB-70 epitopes in C5, suggesting that MPB-83 is more specific to *M. bovis* than MPB-70. Indeed, Alonso and colleagues (2021) compared three bTB antigens (MPB-83, ESAT-6 and CFP-10) and MPB-83 was considered the most specific antigen in the tests. Fresco-Taboada and colleagues (2019) also developed a lateral flow test for identification in wild boar samples, a *M. bovis* wild reservoir, based in MPB-83, with 83% sensitivity and 97% specificity. Based in the results and literature, chimera 5 is a potential MPB-83-containing antigen, perhaps since its disordered structure (MacRaild et al., 2016) with multiepitope repeats facilitates antibody binding, and could be used for further studies, aimed to develop an ELISA and/ or lateral flow rapid serological tests for bTB, with improvements in sensitivity and specificity.

5. Conclusion

Using purified Chimeras 3, 4 and 5 against positive and negative bovine sera for bTB in different immunoassays (indirect dot blot, indirect ELISA, and dot blot in the lateral flow platform), Chimera 5, an MPB-83 epitope containing MCA, had the most promising results and could be used for further studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetimm.2024.110729.

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